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Chemical Kinetic Behavior of Chlorogenic Acid in Protecting Erythrocyte and DNA against Radical-Induced Oxidation

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As an abundant ingredient in coffee, chlorogenic acid (CGA) is a well-known antioxidant. Although some works have dealt with its radical-scavenging property, the present work investigated the protective effects of CGA on the oxidation of DNA and on the hemolysis of human erythrocytes induced by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) by means of chemical kinetics. The inhibition period (t_{inh}) derived from the protective effect of CGA on erythrocyte and DNA was proportional to its concentration, $t_{inh} = (n/R_i)$[CGA], where R_i refers to the radical-initiation rate, and n indicates the number of radical-propagation chains terminated by CGA. It was found that the n of CGA to protect erythrocytes was 0.77, lower than that of vitamin E (2.0), but higher than that of vitamin C (0.19). Furthermore, CGA facilitated a mutual protective effect with VE and VC on AAPH-induced hemolysis by increasing n of VE and VC. CGA was also found to be a membrane-stabilizer to protect erythrocytes against hemin-induced hemolysis. Moreover, the n of CGA was only 0.41 in the process of protecting DNA. This fact revealed that CGA served as an efficient antioxidant to protect erythrocytes more than to protect DNA. Finally, the reaction between CGA and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+\bullet}$) or 2,2′-diphenyl-1-picrylhydrazyl (DPPH) revealed that CGA was able to trap radicals by reducing radicals more than by donating its hydrogen atoms to radicals.

KEYWORDS: Chlorogenic acid; antioxidant; DNA; erythrocyte; hemolysis; hemin

INTRODUCTION

Chlorogenic acid (3-caffeoylquinic acid, CGA, structure in **Scheme 1**) is particularly abundant in coffee, but it is also the principal phenolic acid found in widespread food as sweet potatoes before and after heating treatment (*1*), and its amount is also very important in apples (*2, 3*). The relationship between coffee-drinking and cardiovascular disease has been recently reviewed on the basis of in vitro, cellular, animal and human studies, in which caffeine, polyphenols, and especially CGA have been emphasized on its potential bioactivities (*4*). Thus, the bioavailabilities of CGA derivatives attract much scientific attention. For example, hydroxycinnamic acid was reported to inhibit peroxidation of low-density lipoproteins induced by 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and Cu^{2+} (*5*). However, CGA was also found to be an autooxidant to DNA (*6*), and even was difficult to be absorbed in vivo (*7*). Some researchers pointed out that *m*-coumaric acid, the in vivo metabolite of CGA, makes CGA serve as an antioxidant (*8, 9*). All this background motivates us to investigate the antioxidant properties of CGA, and chemical kinetics used in biological experimental systems may draw a novel conclusion on the antioxidant behavior of CGA.

The present work is composed of three parts. The radical-scavenging property of CGA is first revealed by reacting with two radical species including 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+\bullet}$) (*10*) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (*11*), revealing a different model of CGA to trap $ABTS^{+\bullet}$ and DPPH. Then the protective effects of CGA on hemolysis of human erythrocytes induced by AAPH (*12*) and hemin (*13*) are investigated in order to clarify that CGA protects erythrocyte membranes by trapping radicals and by stabilizing membranes. Meanwhile, the mutual protective effects of CGA and vitamin E (VE) or vitamin C (VC) have also been discussed when VE and VC act as standard antioxidants. Finally, the protective effect of CGA on oxidative damage of DNA induced by AAPH is investigated as well. The original contribution of this work is to apply chemical kinetics for treating the antioxidant results of CGA from biological experiments systems such as erythrocytes and DNA in the case of the peroxidation induced by AAPH.

MATERIALS AND METHODS

Materials. AAPH, ABTS and DPPH were purchased from Fluka, and DNA sodium salt and CGA were purchased from ACROS. Other agents were at analytical grade and used directly. Human erythrocytes were provided by the Red Cross Center for Blood, Changchun, China.

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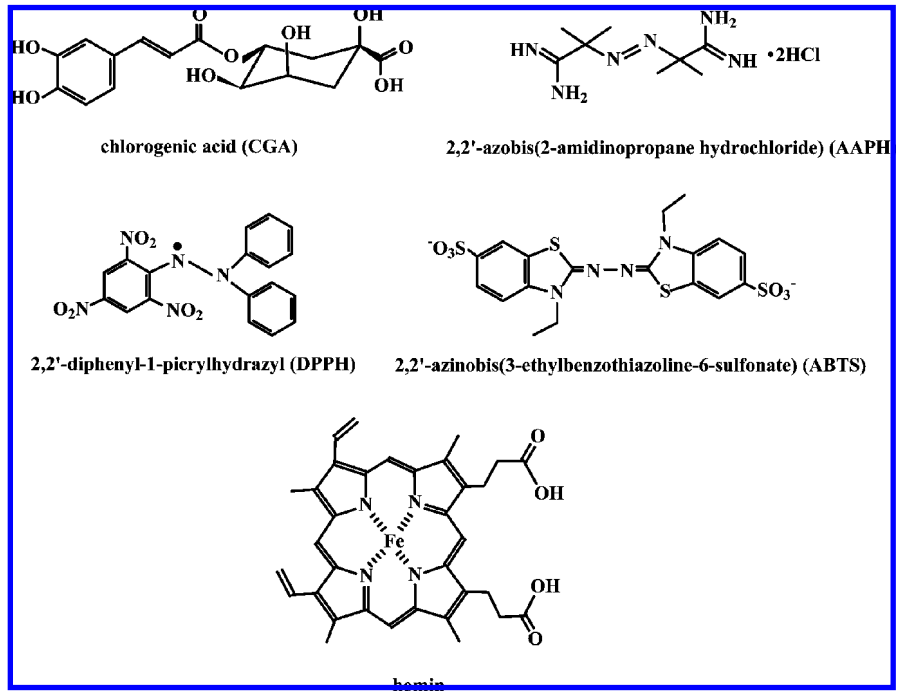
Scheme 1. Structures of CGA, Hemin and Related Radical Compounds Used in This Work



CGA Scavenges $ABTS^{+\bullet}$ and DPPH. The experiments of CGA to scavenge $ABTS^{+\bullet}$ and DPPH were performed following our previous report (*14*). Briefly, the stock solution of DPPH was prepared by dissolving DPPH in ethanol directly to make the absorbance ~1.00 (Abs_{ref}) at 517 nm. ABTS aqueous (4.0 mM, 2.00 mL) was oxidized by 1.41 mM $K_2S_2O_8$ for 16 h, and then diluted with 100 mL of ethanol to make the absorbance ~0.70 (Abs_{ref}) at 734 nm. The addition of various concentrations of CGA aqueous decreased Abs_{ref} to a stable value (Abs_{detect}). Thus, the scavenging percentage of DPPH or $ABTS^{+\bullet}$ in the presence of different concentrations of CGA can be calculated by eq 1.

$$\text{Scavenging DPPH (or ABST}^{+\bullet}\text{) (\%)} = \frac{\text{Abs}_{ref} - \text{Abs}_{detect}}{\text{Abs}_{ref}} \times 100 \quad (1)$$

CGA Protects DNA against AAPH-Induced Oxidation. DNA, AAPH and CGA were dissolved in phosphate-buffered solution (PBSo: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA), respectively. The measurement of AAPH-induced oxidative DNA damage was performed according to the literature (*15*) with a little modification (*16*). Briefly, various concentrations of CGA were added to a mixture of AAPH and DNA, in which the final concentrations of DNA and AAPH were 2.00 mg/ml and 40 mM, respectively. Then the above solution was dispatched into test tubes with 2.0 mL of solution contained in every one. All the tubes were incubated in a water bath at 37 °C to initiate the oxidation. Three tubes were taken out at appropriate intervals and cooled immediately, to which 1.0 mL of thiobarbituric acid (TBA) solution (1.00 g of TBA and 0.40 g of NaOH dissolved in 100 mL of PBSo) and 1.0 mL of 3.0% trichloroacetic acid aqueous were added. The tubes were heated in a boiling water bath for 15 min, and *n*-butanol (1.5 mL) was added and shaken vigorously to extract thiobarbituric acid reactive substance (TBARS). After cooling, the absorbance of the *n*-butanol layer was measured at 535 nm.

CGA Protects Erythrocytes against AAPH- and Hemin-Induced Hemolysis. Human erythrocytes were washed by phosphate-buffered saline (PBSa: 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10 μM EDTA) to remove residual plasma, and centrifuged at 4000 rpm for exactly 10 min at the last washing to obtain compacted erythrocytes (*17*). The hemolysis experimental procedure followed the description in our previous reports (*18*). CGA and AAPH were dissolved in PBSa and added to a 3.0% (v/v) erythrocyte suspension in PBSa (the final concentration of AAPH was 20 mM). The above mixture was incubated at 37 °C to initiate the hemolysis. Aliquots (1.5 mL) were taken out from this mixture at appropriate intervals and centrifuged at 4000 rpm for 5 min to obtain the supernatant whose absorbance (*A*) was determined at 535 nm.

The hemolysis induced by hemin was performed according to our previous report (*19*). Briefly, various concentrations of CGA (dissolved in phosphate-buffered saline without EDTA, PBSe: 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4) were added to a 3.0% (v/v) erythrocyte suspension in PBSe, and hemin (dissolved in 5 mM NaOH to reach 1−2 mM as stock solution) was added to the above suspension at a final concentration of 15.0 μM. Then the above mixture was incubated at 37 °C in a water bath for 30 min, then centrifugated to obtain the supernatant whose absorbance was measured at 535 nm. Finally, the hemolysis percentage (the ratio between the absorbance in the presence and absence of CGA) was plotted vs the concentration of CGA.

Statistical Analysis. All the experiments were carried out independently at least three times to make the experimental error within 10%, and data were expressed as means ± standard derivation. All the quantitative linear relationships in this work were analyzed statistically by one-way ANOVA using Origin 6.0 professional Software, and $p < 0.001$ indicated a significance difference.

RESULTS AND DISCUSSION

CGA Scavenges $ABTS^{+\bullet}$ and DPPH. Figure 1 outlines that the percentages of $ABTS^{+\bullet}$ and DPPH scavenged by CGA are related to the concentration of CGA linearly, which can be expressed by eqs 2 and 3.

$$\text{percentage of ABTS}^{+\bullet}\text{ scavenged by CGA (\%)} = 4.79(\pm 0.15)[\text{CGA}] - 0.13(\pm 1.76) \quad (2)$$

$$\text{percentage of DPPH scavenged by CGA (\%)} = 2.96(\pm 0.04)[\text{CGA}] + 0.16(\pm 0.49) \quad (3)$$

The coefficient in eq 2 (4.79) is larger than that in eq 3 (2.96). This result indicates that more $ABTS^{+\bullet}$ can be scavenged by increasing the concentration of CGA, namely, $ABTS^{+\bullet}$ is more sensitive to the variety of the concentration of CGA than DPPH. Since scavenging $ABTS^{+\bullet}$ reveals the ability of an antioxidant to reduce $ABTS^{+\bullet}$ (*10*), and scavenging DPPH implicates the

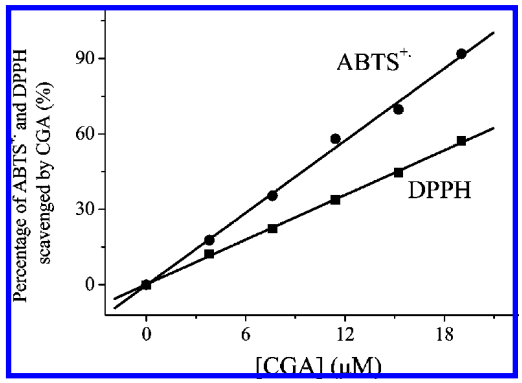


Figure 1. The percentage of $ABTS^{+\bullet}$ and DPPH scavenged by CGA with various concentrations.

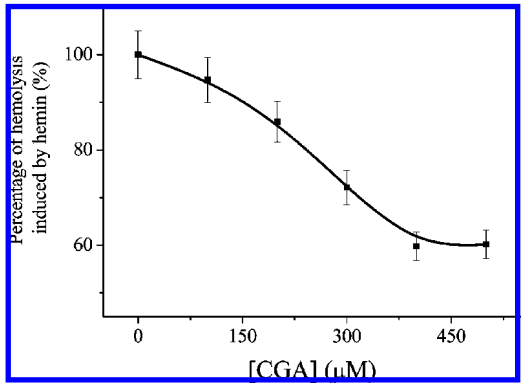


Figure 2. The hemolysis percentage of 3.0% erythrocyte suspension induced by 15.0 μM hemin for 30 min, and inhibited by various concentrations of CGA.

ability of an antioxidant to donate its hydrogen atom to DPPH (*11*), eqs 2 and 3 elucidate that CGA behaves as a reductant more than a hydrogen-donor when it traps radicals.

CGA Protects Erythrocytes against Hemin- and AAPH-Induced Hemolysis. Hemin can destroy the membrane of erythrocytes by accelerating the potassium leakage, dissociating skeletal proteins and prohibiting some erythrocyte enzymes, leading to hemolysis eventually (*20*). An antioxidant can be regarded as a membrane-stabilizer if it protects erythrocytes against hemin-induced hemolysis (*21*). The aim of this research is to identify whether CGA can be a membrane stabilizer in the case of hemin-induced hemolysis of erythrocytes. The absorbance of the supernatant acts as the reference when 3.0% suspension of erythrocytes in PBSe is hemolyzed by 15.0 μM hemin at 30 min. **Figure 2** illustrates that increasing the concentration makes CGA protect erythrocytes against hemin-induced hemolysis remarkably. So, in the case of hemin-induced hemolysis of erythrocytes CGA is proved to be a membrane-stabilizer.

After Niki et al. reported that AAPH can lead to hemolysis of erythrocytes via erythrocyte membrane attacked by peroxyl radicals (*12*), hemolysis mechanism (*22*) and the application of lag time (t_{lag}) in hemolysis to compare the bioactivity of drugs (*23*) attracted much research attention. The original contribution to hemolysis of our group is to express the hemolysis process quantitatively by Boltzmann eq 4 (*24*), and to apply chemical kinetic method to treat the data obtained from hemolysis (*18*).

$$A = (A_{initial} - A_{final})/(1 + e^{(t-t_{lag})/dt}) + A_{final} \quad (4)$$

In eq 4, $A_{initial}$ and A_{final} refer to the absorbance at the beginning and the end of the hemolysis, and t_{lag} stands for the period of

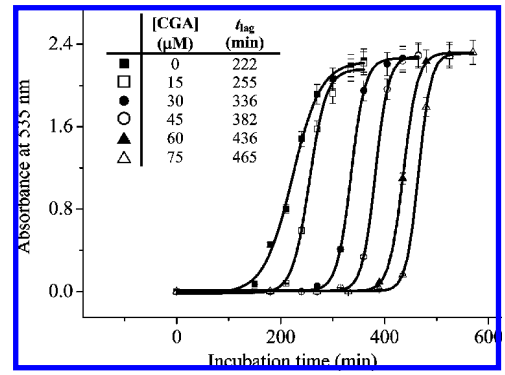


Figure 3. The relationships between the absorbance at 535 nm and the incubation period when 3.0% erythrocytes are hemolyzed by 20 mM AAPH, and inhibited by various concentrations of CGA. All the hemolysis curves are treated by eq 4, and t_{lag} are obtained and listed as the inset table in the chart, where [CGA] = 0 μM serves as the blank experiment.

hemolysis percentage arriving at 50%. The inhibition time (t_{inh}) of hemolysis generated by an antioxidant is designated as ($t_{lag} - t_{lag0}$), where t_{lag0} refers to the lag time in the blank experiment. The t_{inh} eliminates the influence of erythrocytes from different donors (*25*).

Figure 3 illustrates the hemoysis of erythrocytes (3.0% suspension in PBSa) induced by 20 mM AAPH. All the hemolysis curves were regressed by eq 4 via Origin 6.0 professional software to obtain t_{lag} as listed as the inset table in **Figure 3**. The blank experiment is carried out when [CGA] = 0 μM. Then, the t_{inh} is calculated and plotted vs the concentration of CGA as shown as **Figure 5** (vide post). The quantitative relationship between t_{inh} and the concentration of CGA is expressed by linear regression analysis and listed **Table 1**. The linear regression analysis generates a coefficient and a constant consequentially, in which the coefficient reveals the sensitivity of t_{inh} to the variation of [CGA].

It has been reported that, as shown as eq 5, t_{inh} depends linearly on the concentration of antioxidant ([AH]) in a radical-related reaction (*26*).

$$t_{inh} = (n/R_i)[AH] \quad (5)$$

The *n* stands for *stoichiometric factor* to represent the number of radical-propagation chains terminated by one molecule of an antioxidant, and R_i refers to the initiation rate of the radical reaction (*27*). Because it is difficult to measure R_i directly, VE is always selected to be the reference antioxidant whose *n* is taken as 2 (*27*). So, according to our previous report (see **Table 1**) (*18*), R_i can be obtained as 0.224 μM/min when VE is utilized as the standard antioxidant. The *n* of CGA (n_{CGA}) is the product of the coefficient in the equation of $t_{inh} \sim$ [CGA] multiplying R_i, namely, $n_{CGA} = 0.224 \times 3.44(\pm 0.22) = 0.77(\pm 0.08)$, indicating that one molecule of CGA can only terminate less than one radical-propagation chain. Therefore, the antioxidant activity of CGA is lower than VE. Since the *n* of vitamin C (VC) is only 0.19(±0.01) (see **Table 1**) according to our previous report (*18*), the antioxidant activity of CGA is higher than that of VC.

Although orthogonal experimental design is always used to investigate mutual effects between two antioxidants, we investigate the mutual antioxidant effects by increasing the concentration of both CGA and VE or VC. This is because, as shown as eq 6, the influence of [CGA] and [VE] or [VC] on t_{inh} can be expressed as a binary equation with [CGA] and [VE] or [VC] as independent variable (*18*).

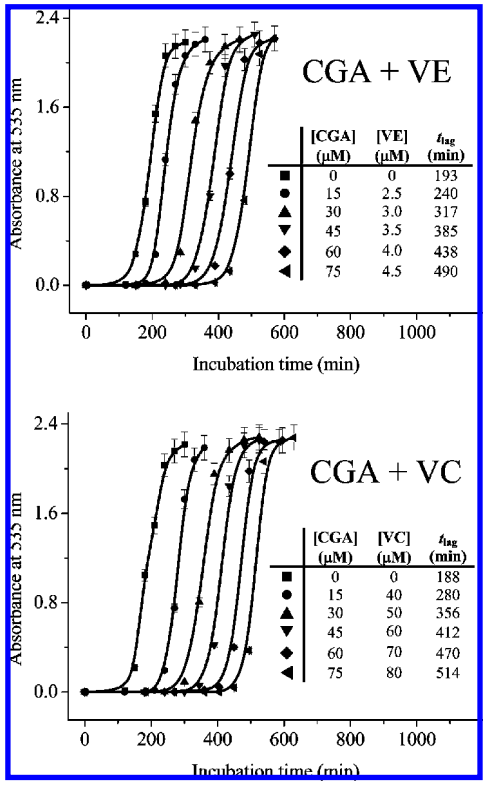


Figure 4. The relationships between the absorbance at 535 nm and the incubation period when 3.0% erythrocytes are hemolyzed by 20 mM AAPH, and inhibited by various concentrations of CGA and VE or VC. All the hemolysis curves are treated by eq 4, and t_{lag} are obtained and listed as the inset table in the chart, where [CGA] = [VE] = [VC] = 0 μM serves as the blank experiment.

Table 1. The Equations of $t_{inh} \sim$ Concentration of CGA when CGA Protects Erythrocytes and DNA Individually or Mutually with VE and VC[a]

$t_{inh} = (n_{CGA}/R_i)$[CGA] + $(n_{VE\ or\ VC}/R_i)$[VE or VC]	n_{CGA}	$n_{VE\ or\ VC}$
In Protecting Erythrocytes		
$t_{inh} = 8.93(\pm1.28)$[VE] − $28.6(\pm18.5)$[b]		2.0[b]
$t_{inh} = 0.83(\pm0.05)$[VC] − $4.02(\pm3.24)$[b]		$0.19(\pm0.01)$[b]
$t_{inh} = 3.44(\pm0.22)$[CGA] − $1.81(\pm9.85)$	$0.77(\pm0.08)$	
$t_{inh} = 1.17$[CGA] + 9.62[VE] + 79.23	0.26	2.15
$t_{inh} = 2.86$[CGA] + 1.51[VC] − 0.08	0.64	0.34
In Protecting DNA		
$t_{inh} = 1.79(\pm0.10)$[CGA] + $21.2(\pm18.5)$	$0.41(\pm0.02)$	

[a] [Erythrocytes] = 3.0%, [AAPH] = 20 mM in hemolysis; [DNA] = 2.00 mg/mL, [AAPH] = 40 mM in the oxidation of DNA. R_i = 0.224 μM/min on the basis of n_{VE} = 2.0 in hemolysis and the oxidation of DNA (*18*). [b] Values of *n* of standard antioxidants involving VE and VC cited from ref *18*.

$$t_{inh} = (n_{CGA}/R_i)[CGA] + (n_{VE\ or\ VC})/R_i)[VE\ or\ VC] \quad (6)$$

Figure 4 illustrates the hemolysis curves in the presence of different concentrations of CGA mixed with VE or VC, and the corresponding t_{lag} is listed in the inset table of **Figure 4**. **Figure 5** illustrates the three-dimensional relationship between t_{inh} and the concentrations of CGA and VE or VC, and the quantitative equations are listed in **Table 1** as well.

Similarly, the coefficient of [CGA] includes the n_{CGA} when CGA functions as a mutual antioxidant with VE or VC, and vice versa. Therefore, the variation of *n* values of CGA and VE or VC implicates the mutual effects of CGA and VE or VC on AAPH-induced hemolysis. Also, on the basis of R_i = 0.224 μM/min, the values of n_{CGA} are 0.26 and 0.64 in the presence of VE and VC,

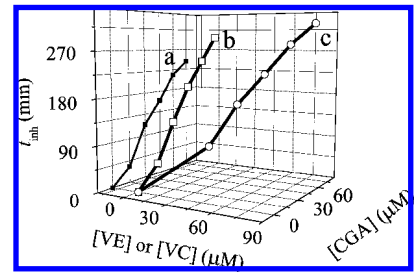


Figure 5. The inhibition period (t_{inh}) is increased by the addition of CGA (**a**), CGA + VE (**b**), and CGA + VC (**c**).

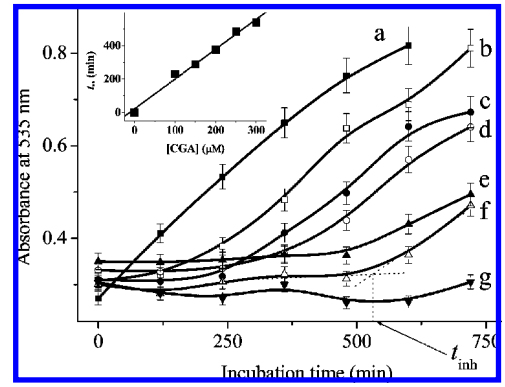


Figure 6. The correlation of the absorbance of TBARS generated from the oxidation of DNA induced by 40 mM AAPH with the incubation time in the presence of 0 μM CGA as the blank experiment (**a**), 100 μM (**b**), 150 μM (**c**), 200 μM (**d**), 250 μM (**e**), 300 μM (**f**) and 500 μM (**g**) CGA. The relationship between the inhibition period (t_{inh}) and the concentration of CGA is illustrated as the inset chart.

respectively, lower than that used individually (0.77). On the other hand, comparing with *n* of VE and VC used individually (2.0 and 0.19, respectively), the values of *n* of VE and VC in the presence of CGA increase to 2.15 and 0.34, respectively, which can be regarded as the contribution from the presence of CGA. So, there exists a mutual antioxidant effect for CGA and VE or VC when they protect erythrocytes.

CGA Protects DNA against AAPH-Induced Oxidative Damage. AAPH is able to convert supercoiled DNA strand into open circular, linear form and more than 20 carbonyl species eventually (*28*), which can be determined quantitatively after reacting with TBA to form TBARS (λ_{max} = 535 nm) (*15*). **Figure 6** outlines the variation of TBARS in the absence and presence of various concentrations of CGA. Line **a** is the blank experiment, indicating that carbonyl species are formed continuously with the incubation time increasing.

With the addition of CGA, TBARS does not form at the beginning of the reaction, and then, the rate of forming TBARS recovers to that in the blank experiment. As a result, t_{inh} is generated as the dotted lines show. Furthermore, as shown as line **g** in **Figure 6**, t_{inh} can even not be observed within the determination period, implicating that CGA protects DNA perfectly when the concentration of CGA is as high as 500 μM. The relationship between the t_{inh} and [CGA] is illustrated as the inset chart in **Figure 6**, and the quantitative equation is also listed in **Table 1**.

We have attempted to determine R_i in AAPH-induced oxidation of DNA by using VE as the reference antioxidant. Unfortunately, VE cannot generate t_{inh} so that R_i cannot be obtained in this case (*16*). Hence, when R_i in AAPH-induced hemolysis (0.224 μM/min) is used to calculate *n* of CGA in protecting DNA, the *n* of CGA is 0.41, lower than that in protecting erythrocytes (0.77). This result demonstrates that CGA protects erythrocytes more efficiently than it protects DNA.

The present result seems contrary to a recent report, in which the oxidative damage of DNA was accelerated by CGA in the presence of Cu(II) ions (*29*). This is because that CGA reduces Cu(II) to form Cu(I), and the Cu(II)/Cu(I) redox cycle catalyzes the oxidation of CGA to form radicals (CGA$^{\bullet}$). Meanwhile, the redox cycle of Cu(II)/Cu(I) leads to the formation of hydroxyl radical ($^{\bullet}$OH). CGA$^{\bullet}$ and $^{\bullet}$OH are able to convert the supercoiled DNA into a relaxed circular form. So, the key step of CGA to be a prooxidant is due to the ability of CGA to reduce Cu(II). This is consistent with our findings in this work that CGA is apt to be a reductant when it reacts with radicals. On the other hand, electrophoresis was applied to follow the oxidation of DNA, whereas the generated TBARS during the oxidation of DNA is measured in this work. Some researchers have pointed out that different determination method may lead to controversy results (*30*).

In conclusion, chemical kinetics is applied to treat the results obtained from hemolysis of erythrocytes and oxidative damage of DNA induced by AAPH and inhibited by CGA. CGA protects erythrocytes more efficiently than it protects DNA, and plays a mutual antioxidant role with VE and VC to protect erythrocytes. CGA is apt to be a reductant more than a hydrogen atom donor when it scavenges radicals. Furthermore, CGA is also a membrane-stabilizer to protect erythrocytes against hemin-induced hemolysis.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt; ABTS$^{+\bullet}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation; CGA, chlorogenic acid; DPPH, 2,2′-diphenyl-1-picrylhydrazyl.

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